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THE EFFECT OF GUINEA PIG TISSUES IN VITRO UPON VIRULENT TUBERCLE BACILLI.

By H. J. CORPER, MAX B. LURIE and O. S. KRETSCHMER.

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It is an almost universally accepted fact that tubercle bacilli are continually being destroyed in the infected animal economy though the mechanism concerned still remains in doubt. There are those who believe the major rôle in this destruction is played by purely immune processes to the exclusion of other mechanisms of resistance, while others lay stress upon the normal cellular powers of resistance such as is displayed by the phagocytic endothelial and other cells. Recently Corper and his coworkers (1) have added another—carbon dioxide—to the possible weapons utilized by the body to hold in abeyance and destroy this insidious invader. In spite of these and many other less significant suggestions regarding the possible mode of inhibition and destruction of the tubercle bacillus *in vivo*, the relative value of these individual factors in this mechanism can only be conjectured and it may be that the really important factor still remains to be discovered. In the ultimate solution and proof of this problem it would simplify matters greatly could the findings be translated by *in vitro* methods. Especially interesting in this respect is the recent report by Webb and his colleagues (2) culminating about four years work on the subject of the fate of tubercle bacilli in lymph nodes. They had found "that when tuberculous lymph nodes, or, for comparison, pieces of liver and spleen are removed from tuberculous guinea pigs, and replanted under the skin in normal animals, and are retained, the bacilli thus transferred invariably produce a general tuberculosis which differs in no apparent way from that which follows

*From the Research Department, National Jewish Hospital for Consumptives, Denver, Colorado.

inoculation with free virulent bacilli. When tuberculous nodes coated with collodion were retained they showed a progressive shrinkage by diffusion through the collodion membrane into the surrounding tissues of the host, until after several months nothing was left of the original node, of about 1 cm. in diameter, but a minute mass about as big as a split pea, enveloped in a shrivelled collodion skin, with its center solidly calcified. In some cases large caseous collodion-coated nodes were planted in tuberculous animals, and this was followed by local inflammation and acute sickness, suggesting that the substance diffusing through the capsule was toxic for the tuberculous animal. But when the coated nodes were planted in normal animals the procedure was followed by no reaction, no altered sensitiveness to old tuberculin, no evidence of change in resistance to a subsequent tuberculous infection, and no lesions of tuberculosis. In one instance a coated node retained for two months and with capsule broken did not produce disease in the guinea pig. Several nodes coated and kept in normal salt solution in the incubator for a few weeks and then inoculated into guinea pigs produced no tuberculosis. Uncoated tuberculous lymph nodes kept in the incubator for fifteen, twenty-nine and forty-three days produced no lesions while the fresh nodes caused a definite tuberculosis developing in the guinea pig within fifteen days. A suspension of tubercle bacilli in saline and kept in the incubator for the same intervals produced tuberculosis when inoculated into guinea pigs. "Subsequent experiences showed that incubated tuberculous nodes had completely lost all power to infect guinea pigs in seven days, and that the same change took place in tuberculous liver and spleen in three weeks or less. The loss of virulence was apparently due to the death of the bacilli as cultures remained sterile in several cases studied. The loss of virulence was not accompanied by any noticeable changes in the shape or staining reaction of the bacilli, though variations occurred, as they often do in fresh material. Apparently, incubated broken or cut open nodes in salt solution produced tuberculosis after thirty-five days incubation. "The following conclusions are drawn from these studies: (1) Tubercle bacilli, enclosed in tuberculous tissue and kept in salt solution at body temperature, lose their virulence and probably die in a few days, regardless of whether contamination occurs or not; (2) Tubercle bacilli free in salt solution, or on the surfaces of crushed tissue, die in a short time when in close contact with certain types of putrefaction, but survive for a long time in the absence of putrefaction." It is suggested that "the most plausible explanation of these facts seems to be that the

bacilli confined in slowly dying tissue, and the bacilli in intimate contact with putrefaction which involves reduction, are deprived of oxygen and soon die from this cause; while bacilli which receive the necessary minimum of oxygen survive for a long time." In a series of communications Bartel and his co-workers from 1905 to 1909 demonstrated that in tuberculous tissues from guinea pigs kept at 37° C. a multiplication of the bacilli was unlikely and a distinct loss of virulence of the bacilli attained within a week (3). According to them the lymphoid organs and lymphocytes played a conspicuous part in this effect. They also found that artificial lymphocytic exudates from animals (dogs) were likewise capable of lowering the virulence of tubercle bacilli and preventing infection in guinea pigs but not to the extent of tissues. Active and inactive serum had no such effect, although it was not entirely inert (4). Newmann and Wittgenstein (5) studied the blood, lung tissue, bronchial and mesenteric glands, liver, spleen and ovaries of dogs and rabbits at different intervals following the intravenous injection of tubercle bacilli into these animals. The tissues were kept in serum to prevent drying out. Blood and all the fresh organs injected immediately after removal revealed bacilli from 30 minutes to 35 days after intravenous injection. The tissues, however, preserved at 37° C. in the incubator destroyed the bacilli, while the blood apparently did not. Bartel and Hartl (6) found that bovine bacilli given intravenously to rabbits also became avirulent in the spleen, mesenteric lymph glands, liver, lung and kidneys *in vitro* at 37° C. while blood possessed no such action. Alkaline thymus decoction (made according to Brieger, Kitasato and Wassermann), even after 54 days had no effect upon tubercle bacilli. Human hydrocele fluid likewise was inactive, the bacilli retaining their full virulence therein (7). The addition of lymph gland decoction to the thymus enhanced the action of the latter. Lymph gland decoction alone had a virulence reducing action. Treatment of guinea pigs with native lymph gland extracts had an inhibitory influence upon the disease in these animals. Realizing, however, the difficulty of having suitable controls for their experiments of long duration, in that the virulence of tubercle bacilli was altered by residence in most so-called indifferent fluids, this point was taken up by them and it was found that after 2 days, distilled water at 37° C. did not affect the virulence, but after 3 days and over there was a perceptible decrease in the virulence of the tubercle bacilli. Three per cent. glycerol added to the water proved a good conserving substance for the tubercle bacilli. This was especially so when added to nutrient solutions, which alone

had a conserving influence upon their virulence; but at 7 days a visible effect was discernible even with these solutions, and at 2 months virulence was lacking. In physiologic saline there was a depression of virulence perceptible in as short a time as 24 hours which was not perceptible after 7 days and was only again perceptible after 2 months (*this is evidently an erroneous observation*), which Bartel attributes to a habituation of the bacilli to the solution. The addition of 3 per cent. glycerol to physiologic saline enhances the deleterious effect, being appreciable at 2 days with complete absence of virulence at 33 days.

Bartel and Newmann (8) and Bartel and Hartl (9) finally used tissues and bacilli for immunizing purposes and obtained therewith beneficial effects in infected guinea pigs. Trudeau and Krause (10) were not able to note any effects of such treatment upon resistance in guinea pigs. They used homologous lymphatic glands and emulsions of tuberculous glands.

Our experiments were performed to gain more chemical information on the destruction of tubercle bacilli by lymph glands, Webb and Ryder and their colleagues having dealt mainly with the biological phases, and if possible to analyze the chemical factors instrumental in destroying the bacilli. The preliminary experiments are more or less a repetition of some of Bartel's and Webb's work but were performed mainly for the purpose of accurate orientation and with this difference that Bartel's and Webb's experiments were performed at 37° C. entirely while in our preliminary experiments room and ice box temperatures were also included. Since the experiments reported which gave the greatest promise of elucidating this phenomenon were the *in vitro* studies, it was decided first to repeat the tuberculous lymph node studies in a systematic manner to note whether the viability of the bacilli was destroyed under different conditions, ice box, room and incubator temperature, and the approximate time required for such action to occur. In addition tuberculous liver and spleen tissues were studied. The tuberculous tissues were obtained from guinea pigs infected with a culture of virulent human tubercle bacilli (Gluckson). The technic consisted of removing aseptically from evident tuberculous animals lymph nodes, pieces of liver and the spleen, placing these intact pieces of tissue into 15 c.c. of sterile 0.9 per cent. sodium chloride solution in a sterile tube plugged with cotton and covered with tin foil to prevent evaporation of the salt solution. The summarized results of this study are given in Table 1.

The findings recorded in Table 1 tend to corroborate those made by Webb and his colleagues in that at incubator temperature tubercle

TABLE I.

The effect of storage of tuberculous tissues under different temperature conditions upon the viability of the contained virulent human tubercle bacilli.

Tuberculous tissue used.	Time interval between removal from animal and testing.	Temperature condition of experiment.	Tuberculosis resulting from the inoculation into a normal guinea pig* after 2 months.		
Lymph node	6 days	Incubator (37.5° C.)	+ p.		0 p.
" "	7 days	Incubator (37.5° C.)	+++ p.	+ p.	++++
" "	7 days	Room (about 20° C.)	+++		+++
" "	8 days	Incubator	0		++
" "	8 days	Room	+++		+++
" "	8 days	Ice box (about 5° C.)		+++	
" "	9 days	Incubator	0	++	+ (6 weeks) †
" "	9 days	Room	+++		+++
" "	9 days	Ice box		+++	
" "	10 days	Incubator	+		++++
" "	10 days	Room	+++		+++
" "	10 days	Ice box		0 (1 week)	
" "	11 days	Incubator	0	++	0
" "	11 days	Ice box		+++	
" "	12 days	Room	++		++
" "	12 days	Ice box		++++	
" "	13 and 14 days	Room	++		++
" "	1 month	Incubator	0		0
Spleen.....	1 month	Incubator	0		0
Lung.....	1 month	Incubator		0	
Lymph node	3 months	Ice box	++++	++++	++++

* The tuberculous involvement resulting two months later from the subcutaneous injection of the tissue after treatment with an equal volume of 3 per cent. sodium hydroxide at 37° C. and washing with sterile saline (Petroff's method of washing for the cultivation of bacilli) is graded from 0, meaning the absence of macroscopic tuberculosis, to ++++, indicating an extensive massive generalized disease. A "p" after the amount of tuberculous involvement means the tissue putrefied. + indicates a definite enlargement of the inguinal nodes with a slight but perceptible enlargement of the retroperitoneal nodes or spleen; ++ indicates an enlargement of the local inguinal and retroperitoneal nodes and visible macroscopic tuberculosis of the spleen; +++ indicates an enlargement of most of the nodes, definite enlargement and involvement of the spleen with visible tuberculosis in the lungs and the liver. These tuberculous gradings are followed throughout this paper.

† The time given in brackets indicates that the test guinea pig died before the two months were over when all the guinea pigs were examined.

bacilli contained in tuberculous tissues tend to lose the power to infect guinea pigs within about 11 days and at least within a month, while at ice box temperature the viability of virulent human tubercle bacilli is retained at least for 3 months in so far as this can be judged from guinea pig infective power. From all present knowledge it would seem that when dealing with virulent human tubercle bacilli, guinea pig infective power is a far better gauge of viability than cultural tests. Cultural tests were made, using the Petroff method, on all the tissues inoculated in Table 1, but with rather irregular results of inconclusive value, so they were not recorded in tabulating the findings. Microscopic studies of the tissues were also made in every case in the hope of being able to find something tangible regarding the lysis of tubercle bacilli but here again the finding bore out in detail the observations recorded by Webb and his coworkers in that they were of negative value only.

In view of the above results it seemed highly desirable to note whether normal tissues inoculated with virulent human tubercle bacilli and treated as in the above experiment would in any way affect the viability of the contained bacilli. For this purpose a very fine suspension of virulent human tubercle bacilli (Gluckson) was prepared and 0.1 milligram in 0.1 c.c. physiologic saline injected into aseptically removed healthy guinea pig tissues, utilizing for this purpose a very fine hypodermic needle (26 gauge) attached to a 1 c.c. tuberculin syringe. The tissue was then placed in a sterile test tube and covered with sterile 0.9 per cent. sodium chloride solution, capped with tin foil to prevent evaporation, and kept for 18 days under different temperature conditions, incubator (37° C.), room (20° C.) and ice box (5° C.). At the end of this time these tissues were ground up, treated for 30 minutes with an equal volume of 3 per cent. sodium hydroxide solution at 37° C., carefully washed to free from alkali, and were then inoculated into guinea pigs and seeded on gentian violet egg medium. Unfortunately normal lymph nodes could not be utilized in this experiment for obvious reasons. The findings obtained two months after injection of the guinea pigs are recorded in Table 2.

The technical difficulties in this experiment were numerous and contamination occurred especially at incubator temperature, but in spite of this it is seen that in the few specimens which proved suitable and remained uncontaminated the results were fairly definite. The tubercle bacilli were destroyed in the liver tissue at both incubator and room temperature but not at ice box temperature, while within 18 days none of the other tissues were able to destroy the bacilli.

TABLE 2.

The viability of virulent human tubercle bacilli inoculated into guinea pig tissues and kept for 18 days under different conditions of temperature.

Temperature.	Tissue inoculated.	Results of inoculation into guinea pig, examined 2 months after infection.*
Incubator	Liver	0
	Lung	++++
	Control (5% acacia saline +0.1 mg. tubercle bacilli)	+++
Room	Liver	0
	Spleen	+++
	Testes	++
	Control	++ and +
Ice Box	Liver	+++
	Spleen	+++
	Lung	++
	Kidney	+++
	Testes	+++
	Control	+++

* The macroscopic grade of tuberculosis found in the guinea pigs inoculated is given from 0—no visible tuberculosis to ++++— a massive generalized disease. (See foot note to Table 1.)

Thinking it possible that tissues *in vitro* gradually developed substances possessing toxicity for virulent human tubercle bacilli an experiment was planned to test this point. For this purpose tuberculous lymph nodes, and tuberculous liver and spleen from guinea pigs (infected with Gluckson) were removed with all aseptic precautions possible, suspended in sterile 0.9 per cent. sodium chloride solution, and placed in the incubator for 14 days, at the end of which time they were removed from the saline solution, ground up fine in a meat grinder with aseptic care as far as this was possible. The tuberculous lymph gland tissue, ground up, and without dilution was placed in a sterile tube and to it was added a very fine suspension of virulent human tubercle bacilli (Gluckson) so that each 1 c.c. of ground tissue contained 0.01 mg. of bacilli (0.000,000,01 mg. of this bacillus was sufficient to produce a generalized tuberculosis in 2 months), and the entire well mixed to insure a fairly uniform distribution of the bacilli in the ground-up tissues. The liver and spleen tissues mixed, were treated in a like manner and the same proportion of the virulent human tubercle bacilli added. Smears made and examined microscopically after staining revealed a goodly number of bacilli, many more than were present in the original ground tuberculous tissues before the addition of the 0.01 mg. of suspension. The mixtures were

then placed, in 1 c.c. amounts, in small sterile test tubes and were superimposed with a thin layer of liquid petrolatum. At intervals of from 1 hour to 8 days individual tubes were removed and after sodium hydroxide treatment and washing (Petroff's method), were utilized for guinea pig inoculation to determine the viability of the bacilli contained therein. The findings in the guinea pigs, 2 months after inoculation, are recorded in Table 3.

TABLE 3.

The effect of incubated (14 days) tuberculous lymph nodes, mixed tuberculous spleen and liver tissues upon virulent human tubercle bacilli at 37° C.

Incubated (14 days) intact tuberculous tissue tested.	Interval between mixing of ground tissue with bacilli and inoculation for testing.	Tuberculosis found in the guinea pigs 2 months after inoculation of the ground tissues and suspension of bacilli.*
Lymph gland	1 hour	++
Spleen and liver	1 hour	++
Lymph gland	4 hours	++
Spleen and liver	4 hours	++
Lymph gland	18 hours	0
Spleen and liver	18 hours	0
Lymph gland	24 hours	0
Spleen and liver	24 hours	0
Lymph glands	2 days	0
" "	3 days	0
" "	4 days	0
" "	5 days	0

* The macroscopic tuberculosis is here graded from 0 = none visible, to ++++ = a generalized massive involvement, as detailed in Table 1.

The results obtained from the experiment recorded in Table 3 reveal that tuberculous lymph nodes and mixed tuberculous liver and spleen tissue removed from a guinea pig infected with virulent human tubercle bacilli and kept intact in sterile 0.9 per cent. sodium chloride solution in the incubator for 14 days undergo changes coincident to such incubation so that when subsequently ground up and mixed with relatively large amounts (0.01 mg. per 1 c.c. of ground tissue) of fine suspensions of virulent human tubercle bacilli they are able to so change these within 18 hours at 37° C. as to make them incapable of infecting normal guinea pigs.

If tuberculous tissues kept in the incubator for 14 days are thus capable of destroying the infective power of virulent human tubercle bacilli, the question naturally arises whether normal tissues possess or are devoid of this same property. To test this another experiment was planned of similar nature to the above, but including, besides controls in 0.9 per cent. sodium chloride solution, normal spleen, nor-

mal liver, and for comparison tuberculous lymph node and tuberculous spleen from guinea pigs. The tissues were placed in the incubator for about ten days, after which they were ground up aseptically, filtered through several thicknesses of sterile cheese cloth, and the filtrate mixed with a suspension of virulent human tubercle bacilli (0.01 mg. in 0.1 c.c. to 1 c.c. of tissue filtrate). The mixture with contained bacilli was placed in small sterile test tubes in 1 c.c. amounts and superimposed with a small amount of pure sterile liquid petrolatum and placed in the incubator. The controls being treated in the same manner consisted of 1 c.c. of 0.9 per cent. sodium chloride solution containing 0.01 mg. of tubercle bacilli with superimposed liquid petrolatum. At definite intervals a tube of each specimen was taken from the incubator, treated with an equal volume of 3 per cent. sodium hydroxide solution for 30 minutes at 37° C., washed well with sterile 0.9 per cent. sodium chloride solution to free from alkali, and the sediment was injected subcutaneously into normal guinea pigs. The guinea pigs were examined macroscopically for tuberculous involvement 2 months after injection. The findings are recorded in Table 4.

TABLE 4.

The effect of incubated normal and tuberculous guinea pig tissue—filtrates upon virulent human tubercle bacilli at 37° C.

Tissue-filtrate used.	Interval of contact with virulent tubercle bacilli.	Tuberculosis produced in normal guinea pigs 2 months after injection of the filtrate and virulent bacilli.
Saline control	20 hours	++++*
Normal spleen	" "	0
Normal liver	" "	++
Tuberculous lymph node	" "	0
Tuberculous spleen	" "	0
Saline control	48 "	++++
Normal spleen	" "	0
Normal liver	" "	0
Tuberculous lymph node	" "	0
Tuberculous spleen	" "	0
Saline control	3 days	+++
Normal spleen	" "	0
Normal liver	" "	0 (1.5 month)
Tuberculous lymph node	" "	0
Tuberculous spleen	" "	0
Tuberculous liver	" "	0

* The tuberculous involvement found in the guinea pigs 2 months after inoculation is graded from 0 = no visible macroscopic involvement, to ++++ = a massive generalized tuberculosis of practically all the lymph glands and important organs as in the previous three tables.

The experiment recorded in Table 4 would make it seem highly probable that during the incubation of normal or tuberculous guinea pig tissues *in vitro* there is formed a product, probably in water-soluble form, of toxic nature toward virulent human tubercle bacilli and capable of destroying their infective power for guinea pigs within a comparatively short time, 20 hours at 37° C. The toxic substance does not seem to be of the nature of an acid, expressed as hydrogen-ion concentration, since tubercle bacilli, both human and bovine, will grow in a medium with a pH value less than that which was noted in these tissue filtrates. This will be considered more fully in the following experiments of this paper. It is noteworthy also that in so far as the intactness of the morphology and staining properties of the human tubercle bacilli was concerned, no apparent change could be noted even after 4 months incubation in intact tuberculous lymph nodes or in ground up tissues of any of the kinds studied.

In the previous experiments recorded in this paper only the extracts from normal spleen and liver were tested for their toxic action on tubercle bacilli. In order to extend the observations to the more important other organ tissues, the observations recorded in Table 4 were extended to lung, muscle (voluntary), brain, heart and kidney, besides liver and spleen tissues. The aseptically obtained tissues were incubated with saline for 14 days, covered with liquid petrolatum, ground up at the end of this time, the clear liquid portion of the ground tissues obtained by centrifugation of the stoppered tubes at 3000 r. p. m. for a considerable time, and tubercle bacilli (0.16 mg. per c.c.) added, as in the previous experiment, and the mixture incubated at 37° C., a portion (0.25 c.c., larger amounts than this being fatal to the guinea pig) being drawn off at different intervals, to be injected into guinea pigs, which were examined two to three months later to determine whether the bacilli had lost their viability. The findings are recorded in Table 5.

The findings recorded in Table 5 indicate that clear tissue filtrates prepared from incubated organs from guinea pigs are able to abolish the virulence of tubercle bacilli at incubator temperature, this ability, however, varying for the different organs tested. Voluntary muscle filtrate was powerful enough to accomplish this within one day; liver, lung, spleen, within 4 days; brain, heart, muscle and kidney tissue only after one week. It is noteworthy also that the hydrogen-ion concentration of the filtrates bore no relation to the ability of the filtrate to destroy the virulence of the tubercle bacilli; muscle filtrate, the most active tissue filtrate, having a pH of about 6.5, as compared

TABLE 5.

Tuberculosis in guinea pigs injected with clear tissue filtrates, prepared from normal guinea pig organs previously incubated with saline 14 days, mixed with suspensions of tubercle bacilli and kept at 37° C.

Organ.	Specimen.	Time interval of incubator residence between mixing of tissue filtrate with suspension of tubercle bacilli and subcutaneous inoculation of mixture into test guinea pig.								Hydrogen ion concentration (pH) of tissue filtrate.
		1 hour.	1 day.	2 days.	3 days.	4 days.	1 week.	2 weeks.		
Liver.....	*a*	+++	+++		0	0	0		0	4.63
	b	+++	++		0–1 mo.	0	0		0	4.56
	c	+++	++		++ 6 ws.	0	0		0	4.68
	d			+++		0				
	e			+++*		0–9 ds.		0		
	f			0–7 d.		0		0		
Lung.....	*a*	+++	0–3 ds.		+++	0	0		+++	7.86
	b	++++ 6 w.	+++		0	0	0		0	7.57
	c			+++		0–12 ds.		0		
	d			0–1 d.		0		0		
	e			0		0		0–5 ds.		
Muscle.....	*a*	++	±		0	0	0		0	6.58
	b	+++	0		0	0	0		0	6.70
	c			0		0–1 mo.				
	d			lost		0		0		
	e			0–10 ds.		0		lost 0		

* The letters "d," "w" or "mo," after the numeral, indicates the number of days, weeks or months respectively after which the animal died following injection.

TABLE 5—*Continued.*

Organ.	Specimen.	Time interval of incubator residence between mixing of tissue filtrate with suspension of tubercle bacilli and subcutaneous inoculation of mixture into test guinea pig.								
		1 hour.	1 day.	2 days.	3 days.	4 days.	1 week.	9 days.	2 weeks.	Hydrogen ion concentration (pH) of tissue filtrate.
Spleen.....	*a*	+++	±		0	0–7 ds.	0		0	6.65
	b	+–2 ws.	± 1 mo.		+	0–7 ws.	0		0	
	c			0—9 ds.		0–2 ds.		0		
	d			0–11 ds.		0				
Brain.....	*a*	++++	+++		++++	± 3 ws.	++		0	7.21
	b			0–3 ds.		+		0		
	c			0–7 ds.		0–5 ds.		0		
Heart.....	*a*	+++	± 2 ws.		++++	0–8 ds.	±		0–3ds.	7.84
	b	+++ 1 mo.	++++		+++	± 1 mo.	+++ 7 ws.		0	5.62
	c			0–2 ws.		0–7 ds.		0		
	d			0–9 ds.		+++		0		
Kidney.....	*a*	+++	++ 1 mo.		+++	++	+++		0	5.88
	b	++++	± 1 mo.		0–3 ws.	0–12 ds.	+++		0–10 ds.	
	c			±		0–2 ws.				
	d			0–4 ds.		0–3 ds.		0		
Control.....		+++	++	+++	+	+++	++	+	++	
		++++		+++	+++	++		++	+	
						+++				

to brain pH 7.2, one of the least active, and liver pH 4.6, acting about the same as lung pH 7.6. Recently Sevringhaus (11 and 12) has been studying postmortem acidity and his figures for autolyzing liver indicate that a high degree of acidity is never reached, in that a pH 6.0 may be reached in some cases only, the acidity never exceeding this for this tissue according to him. He used pork and dog livers, but the exact temperatures at which autolysis was carried out is not clear.

Believing the tuberculotoxic substances formed by the tissues during incubation to be due to aseptic autolysis of the organs and that probably similar substances would form during antiseptic autolysis, it was planned to incubate the same organs, liver, lung, heart, voluntary muscle, kidney, spleen and brain, for fourteen days in sterile 0.9 per cent. sodium chloride solution in the presence of chloroform and toluene as antiseptics, prepare a tissue filtrate from each, remove the chloroform and toluene, add the tubercle bacilli, incubate the mixture and test for the virulence of the bacilli as in previous experiments at definite intervals by inoculating guinea pigs. Chloroform and toluene were chosen as antiseptics because they were volatile, had been repeatedly used in autolysis studies, and could be completely removed by passing a current of sterile air through the solution before adding the bacillus suspension. The results of this experiment are recorded in Table 6.

In order to note whether thermolabile phenomenon were being dealt with, in the action of filtrates derived from the incubated tissues upon the tubercle bacilli, the same organs were heated for one hour in a boiling water bath before the primary incubation for fourteen days, after which the tissue filtrate was prepared with sterile precautions, the suspension of tubercle bacilli added (0.16 mgs. per c.c.) and after definite intervals of residence at 37° C. in the incubator a portion of the mixture was injected into guinea pigs. The results of the guinea pig inoculations with the mixture 2 months later are recorded in Table 7. The findings recorded in Table 6 were rather unexpected in that the tuberculo-toxic substances were not produced by normal tissues during antiseptic (in the presence of chloroform and toluene) autolysis at incubator temperature within fourteen days and required further elucidation. It was thought, however, that the depression of autolysis due to the presence of the antiseptics may not have permitted the process to progress sufficiently within the fourteen days and that longer incubation may still result in positive findings. This, however, did not prove to be the case, since tissue extracts, prepared from guinea pig voluntary muscle and liver which had been incubated 45 days

TABLE 6.

Tuberculosis in guinea pigs injected with the clear tissue filtrates, prepared from normal guinea pig organs incubated with saline and chloroform and toluene, as antiseptics, for 14 days, mixed with suspensions of tubercle bacilli and kept at 37° C.

Organs.	Tissue specimen.	Time interval of incubator residence between mixing tissue filtrate (aerated to free from chloroform and toluene) with suspension of tubercle bacilli and subcutaneous inoculation into test guinea pig.						Hydrogen ion concentration pH: of tissue filtrate.
		1 hour.	1 day.	2 days.	5 days.	1 week.	2 weeks.	pH
Liver.....	*a*	+++	++++	++++	+++	+++ 1 mo.	+++	4.59
	b	++++	++ 1 mo.*	++++	+++	++++	+++	
Lung.....	*a*	+++	++++	+++	++ 6 ws.*	+++	+++	7.01
	b	+++	++++	0–3 ds.	0–2 ds.	++++	+++	
Heart.....	*a*	+++ 1 mo.	++++	± 5 ws.	+++ 5 ws.	+++	0–1 mo.	7.35
	b	++++	++++	0–2ds.	+++ 5 ws.	+++	+++	
Muscle.....	*a*	+++	0–3 ds.	0–4ds.	++++	0–3 ds.	± 1 mo.	5.67
Kidney.....	*a*	lost	++++	+++ 5 ws.	+++	0–3 ds.	++	6.63
	b	++++	++++	++++		± 1 mo.	± 1 mo.	6.68
Spleen.....	*a*	+++ 1 mo.	++	0	0	0	±	4.26
Brain.....	*a*	+++	++++	+++	+++	++++	+++	7.85
	b	++++	++++	++ 1 mo.	+++		+++	7.41
Control.....		+++	+++ +++ 1 mo.	++++	+++	+++	0–6 ws. +++	

* All the guinea pigs were examined two months after injection except as otherwise indicated.

The letters "d," "w" or "mo.," after the numeral, indicates the number of days, weeks or months respectively after which the animal died following injection.

TABLE 7.

Tuberculosis in guinea pigs injected with the clear tissue filtrates, prepared from normal guinea pig organs in saline, heated one hour in a boiling water bath and incubated for 14 days, mixed with suspensions of tubercle bacilli and kept at 37° C.

Organ.	Tissue specimen	Time interval of incubator residence between mixing tissue filtrate with suspension of tubercle bacilli and subcutaneous inoculation into test guinea pig.			Hydrogen-ion concentration (pH) of tissue filtrate.
		1 hour.	1 day.	2 weeks.	
Liver	*a*	++++	++++	0	
	b	++++	+++	0	4.96
	c	+++	++++	+++	5.64
Lung	*a*	++++	++++	+++	
	b	++1 mo.*	++++	++	6.41
	c	+++	++++	++	7.59
Heart	*a*	++++	++++	++++	7.05
	b	++++	0–1 mo.	++++	
	c	++++	+++	+++	7.50
Muscle	*a*		+++1 mo.	++	
	b	++++	0	++	4.90
	c	+++1 mo.	+++1 mo.	0–1 mo.	
Kidney	*a*	++1 mo.			
	b		++++	±	
	c	+++	++++	++	7.04
Spleen	*a*	+++	++++	+++	5.98
	b	+++1 mo.	+++	+++	4.76
	c	+1 mo.	++++	+++	
Brain	*a*	++1 mo.	+++	+++	7.42
	b	++++	++++	++++	
	c	++++	++++		7.26
Control		++++	++++	+++	
		++++	+++		

* "mo" indicates the number of months after which the animal died following injection.

after the addition of toluene and chloroform, when freed from antiseptics by aeration and incubated with virulent human tubercle bacilli, did not prevent their infecting guinea pigs even after 9 days incubator residence. It seems from these experiments that the antiseptics toluene and chloroform either so retard autolysis or alter it at incubator temperature that the tuberculo-toxic substances are either not found in sufficient amounts to be discernible by the method of testing used or are destroyed as rapidly as they are produced under these conditions. This would be analogous to the findings which obtained at room and ice box temperatures. The effects with boiled

normal tissues recorded in Table 7 were as expected and resulted entirely negatively, thus indicating that the soluble tuberculo-toxic substances originated from the tissues during their residence in the incubator.

In view of the fact that heating to the boiling point of water before incubation destroyed those elements concerned in the formation of the tuberculo-toxic substances from normal tissues at incubator temperature, it seemed desirable to determine whether the toxic substances once formed were thermostabile and were non-coagulable by heat.

For this purpose five organs of the guinea pig were used, voluntary muscle, liver, lung, spleen and brain. The experiment was performed in two series. In the first series (specimens a and b) the tissues after incubation for fourteen days in saline solution at 37° C., were ground up with aseptic precautions and the clear tissue filtrates obtained therefrom. One portion was utilized as such, being mixed with a suspension of virulent human tubercle bacilli (0.16 mg. of "Gluckson" to 1 c.c. of filtrate), and after four and eight days incubator residence at 37° C. was tested by guinea pig injection, while another portion of the same filtrate was first heated for one hour in a boiling water bath, the coagulum separated and the clear filtrate therefrom cooled and mixed with the same suspension of tubercle bacilli and kept exactly as the first portion, and tested after four and eight days incubator residence by injection into guinea pigs. The second series (specimens 1, 2 and 3) is a duplicate of the first series, but it was performed separately and at a subsequent date. The results of the guinea pig inoculations for series I and II are recorded in Table 8.

An examination of Table 8 indicates that the tissue filtrates from guinea pig muscle, liver and spleen, prepared from normal tissues incubated for fourteen days, heated in a boiling water bath for one hour, so that the coagulable proteins were removed, were still capable of abolishing the guinea pig infective power within four to eight days at incubator temperature. Although it is possible that some of the tuberculocidal potency was removed with the coagulable protein portion of the toxic filtrate the greater part was still retained in the non-coagulable filtrates from these organs.

Since it was found that antiseptic (chloroform, toluene) autolysis of normal tissues for 14 days at incubator temperature did not yield the tuberculo-toxic substances, at least in concentration equal to that obtained from tissues incubated without the addition of antiseptics, and aeration had been used to free the filtrates at the end of this time from the antiseptics before adding the suspension of tubercle bacilli,

TABLE 8.

Tuberculosis in guinea pigs injected with heated and unheated clear tissue filtrates, prepared from normal guinea pig organs in saline incubated 14 days, mixed with suspensions of tubercle bacilli and kept at 37° C. for 4 and 8 days.

Normal guinea pig organ used.	Series.‡	Normal, unheated, tissue filtrate, prepared after 14 days incubation, mixed with tubercle bacilli, kept at 37° C. and tested after:		Normal, heated, tissue filtrate, prepared after 14 days incubation, the coagulable protein-free filtrate mixed with tubercle bacilli, kept at 37° C. and tested after:	
		4 days.	8 days.	4 days.	8 days.
Muscle.....	(*b*)*	0 4 ws.†	0	0	0
	(1)	0	0	0	0
	(2)	+++	0	0	0
Liver.....	(*a*)	0	0	+++	+++
	(*b*)	+++	++	+++	+++
	(1)	0	0		
	(2)	0	0	0	0
	(3)	+++	++	+++	0
Lung.....	(*a*)	++	+	0 died (5 ds.)	++
	(*b*)	0	0	+++	++
	(1)	++	++	++	0
	(2)	+++	++	++	++
	(3)	+++	++	++	0
Spleen.....	(*a*)	0 7 ws.	0	0	0
	(*b*)	+++	+	+++	died (12 ds.)
	(1)	0	0	0	0
	(2)	0	0	++	+
	(3)	0	0	++	+
Brain.....	(*a*)	+ 5 ws.	++	++	++
	(*b*)	+++		++++	+++
Control: Saline.....	(*a*)	++++	+++		
	(*b*)	++++	+++		
Control: Boiled liver filtrate....	(1)	+++	++		
	(2)	+++	++		
	(3)	++	++		

* The small letters (*a*) and (*b*) indicate that the tissues used were two separate specimens of either liver, lung, etc., or in the case of the control two separate saline solutions were used, and that the two guinea pigs recorded were not injected from the same specimen.

† The amount of tuberculosis found in the guinea pigs is graded as in previous tables from 0 to ++++. All examinations were made 2 months after infection unless the time is otherwise designated in the bracket following the tuberculosis grading.

‡ The letters under this column indicate that the tests were conducted as series I, while the numerals indicate that the tests were conducted at a later date, as series II.

there entered the possibility that the tuberculo-toxic substances were of volatile nature and were removed during the process of aeration. To test this, however, two tissues were chosen, viz., guinea pig liver and voluntary muscle. They were incubated for 14 days without antiseptics and after grinding, the filtrates were divided into two portions, one portion retained as control and the other aerated in identical manner to that used with the previously recorded antiseptic autolysates. Suspensions of living tubercle bacilli were then added to both filtrates, the aerated and non-aerated controls, they were kept at incubator temperature and after 4 and 8 days residence at 37° C., guinea pigs were injected with equal amounts of each. The results of the injections are recorded in Table 9.

TABLE 9.

The effect of aeration upon the tuberculo-toxic substances in the filtrates prepared from normal guinea pig tissues (liver and muscle) after 14 days incubation.

Normal guinea pig tissues used.	Treatment of tissue filtrates before mixing with tubercle bacilli and final residence in the incubator prior to injection into guinea pigs.			
	Control tissue filtrates (not aerated) incubated with bacilli for		Tissue filtrate aerated prior to incubation with bacilli for	
	4 days.	8 days.	4 days.	8 days.
Liver	++++*	0	0	0
Muscle	0	0	0	0
Fresh boiled (not incubated) liver filtrate control	+++	+++		
Fresh boiled (not incubated) muscle filtrate control	++++	++		

* The tuberculosis gradings conform to those in previous tables.

The findings recorded in Table 9 indicate clearly that the tuberculo-toxic substances are not of volatile nature, at least in such form that they are removed by prolonged aeration which has been found capable of removing the contained chloroform and toluene, used as antiseptics, from the tissue filtrates.

Discussion

Certain phenomena occurring within the body such as the liquefaction of dead tissues without putrefaction, first alluded to as due to the effects of digestive ferments by Hoppe-Seyler in 1871, initiated

studies which resulted in Salkowski's demonstrating that this softening of dead tissues was brought about by a true digestion by intracellular enzymes—"autodigestion." Little attention was paid to this work, however, until Jacoby in 1900 renamed the process autolysis (13). The greatest strides in the study of this subject have been made since this time and by utilizing the Salkowski method, depending upon the difference in the suceptibility of bacteria and of enzymes to antiseptics. In much of the work recorded use has been made of toluene and chloroform as antiseptics, in spite of the fact that these antiseptics exert acknowledged influences upon the process which have been considered only of quantitive significance, although it is admitted that qualitative differences are possible. The term autolysis is not restricted to the use of antiseptics, but organs obtained aseptically may be submitted to aseptic autolysis in contradistinction to antiseptic autolysis by the Salkowski method. It is now acknowledged that proteases are present in every cell of the body, but that the rate of digestion differs in different organs, liver digesting rapidly, while brain and muscle digest more slowly and the rate varies under different conditions. So far as we are aware from the literature there has been no correlation between the rate of digestion of the various organs or the conditions under which digestion was carried on and the toxicity to the tubercle bacillus.

Bradley (14, 15, and 16) and his colleagues have found that the hydrogen-ion concentration plays an important part in accelerating or retarding autolysis by so modifying the substrate that the enzymes can attack it; a small excess acidity may, however, destroy the enzymes, the maximum autolysis being obtained at about pH 6.0, while pH 7.4–7.8 reduces autolysis to a minimum. Brain tissue according to Gibson, Umbreit, and Bradley (17) autolyzes in the same way as other tissues, though quantitatively on a smaller scale, the speed and extent being dependent upon the H-ion concentration, in alkaline or neutral media being inhibited. The intracellular proteases are not identical with pepsin or trypsin (18), and the changes during autolysis are not limited to the proteins; glycogen and the sugars are split, fats are split, reducing substances appear and volatile fatty acids form besides carbon dioxide, volatile acids and hydrogen; creatinine and choline form besides many amino acids and purine bases.

In considering the toxicity of tissue autolysates to animals or lower forms of life, it is well to be aware of the fact that native tissue extracts which have not been subjected to autolysis may possess toxic properties under certain circumstances. The greater part of the

recent literature concerns itself with the intravenous injection of lung extracts in which case intravascular clotting due to tissue coagulins complicates the phenomenon (19 and 20). Boiling, however, makes the tissue extracts innocuous (21), which is not the case with the autolysates. Brieger and Uhlenhuth (22), as early as 1898, reported that tissue extracts from guinea pig, man and other animals, given subcutaneously caused death in guinea pigs, the toxicity of brain, liver, kidney, lung, adrenal, spleen, lymph gland and bone marrow being about equal; this has since then been noted frequently by many laboratory workers who are in the habit of injecting untreated diseased tissues or sputum into animals, especially guinea pigs, for diagnostic purposes. However, for the tubercle bacillus the native toxicity of fresh tissues, as has been shown in this study, is practically nil, while in sputum the bacilli may live many days uninjured. There is, therefore, a distinct difference between the toxicity of these substances for the animal economy and for the tubercle bacillus. It would seem from *a priori* reasoning that the tubercle bacillus is more resistant to the tissue disintegration products than the animal, although it must be admitted that the conditions are not quite comparable, the factors involved in the animal being far more complicated. It is common knowledge that whole or ground up homologous or heterologous fresh tissues, injected subcutaneously, lead to the formation of large abscesses and ulcers, probably the result of the action of toxic products liberated by the combined action of autolytic enzymes and the natural body enzymes, although inhibitory (anti-enzyme) influences may be operative in this phenomenon. It is not certain whether the enzymes differ or whether anti-enzymes are operative (23). It is interesting in this connection to note that Kraus and Volk (24) found extracts of tuberculous organs from guinea pigs toxic, when given intravenously to normal guinea pigs, while similar extracts from normal organs (kidney, adrenal and brain) were inactive. Little attention need be paid to bacterial autolysis here, although this may be significant in the case of rapidly growing bacteria, (25), since the tubercle bacillus autolyses very slowly at 37° C. (26), and within the comparatively short periods of time concerned here could hardly liberate products of sufficient potency to exert a detrimental influence upon the bacilli themselves (27).

The suggested oxygen deprivation, as an explanation for the effect of the tissues *in vitro* upon the tubercle bacillus, is hardly tenable for many reasons. If such were the case the bacilli should lose their virulence in boiled normal tissues in which the boiling would tend to

deprive the mixtures from free oxygen, or in view of the fact that reductases may be instrumental in the oxygen deprivation and these be destroyed by heating, the bacilli should not lose their virulence in the heated filtrates free from coagulated protein prepared from tissues previously incubated for fourteen days. This is contrary to the facts. Also many of the controls which failed to abolish the virulence were prepared under liquid petrolatum and from boiled solutions, the latter procedure tending to remove all free oxygen, while in some combined oxygen could hardly be considered to be present. In using the term reductases it is realized that the existence of enzymes of this nature may still be questioned.

An interesting report pertinent to the subject under discussion was contributed years ago by Conradi (28) who found that bactericidal substances developed during the autolysis of animal (organ) and plant (yeast) cells. Aseptic autolysis proved far more serviceable in the production of the bactericidal substances than antiseptic (chloroform and toluene) autolysis. The latter procedure had a marked inhibitory influence upon the development of the bactericidal substances. In certain cases prolonged aseptic autolysis also resulted in a depreciation of the toxic substances. Short interval aseptic autolysis proved preferable with dysentery and typhoid bacilli. The toxic substances were water soluble (29). Lymph glands, liver, spleen, bone marrow, thymus, adrenal, testicle, muscle, intestinal walls, lung, tonsils, ovary, pancreas, brain and kidney of the cow were tested after autolysis, using only the water soluble filtrate, against a large variety of rapidly growing bacteria (anthrax, typhoid, cholera, staphylococci, proteins, pyocyaneus, Friedlander's and diphtheria bacilli). Of these, pancreas, brain and submaxillary gland developed no bactericidal substances; of the fresh organs tested only lymph glands yielded active extracts. Regarding the nature of the toxic substance it is pointed out that it is not known whether it is a single substance or a mixture of substances, or whether the active substance differs in the various organs tested. It is believed that the differences found are only quantitative. The toxic extract from the liver was not destroyed by four to five hours heating to the boiling point; this rather enhanced the bactericidal properties. It passed a Chamberland filter unaffected; charcoal, lycopodium or starch had no effect upon it. The substance is diffusible, alcohol soluble and is precipitated by the further addition of ether. It gives the protein reactions and is probably the aromatic part of the protein molecule (30).

The experiments recorded in this contribution on the effect of tissue

autolysates upon the tubercle bacillus seem to agree in almost all particulars with the findings obtained by Conradi in using the organs from the cow and studying the effect of the products of autolysis upon the rapidly growing microorganisms. It seems that the effect of animal (guinea pig) tissues upon the virulence of tubercle bacilli during residence in the incubator, as noted by Webb and Bartel, is best attributed to the development of tuberculo-toxic substances by these tissues at this temperature. If there are other factors concerned in this action they would seem from these observations to be only subsidiary and of less importance.

Summary.

Virulent human tubercle bacilli, contained in tuberculous lymph nodes, removed from guinea pigs and kept in sterile physiologic sodium chloride solution at incubator temperature, lose their infective power for guinea pigs in less than two weeks in corroboration of the findings reported by Webb, Ryder and Gilbert. At room (20° C.) and ice box (5° C.) temperature the tubercle bacilli remain unaffected for a longer time, for at least 3 months in the latter case. During 4 months residence in the incubator in intact tuberculous lymph nodes the human tubercle bacilli suffered no perceptible microscopic morphologic changes. During incubation of normal or tuberculous guinea pig tissues *in vitro* there is found a product within two weeks, in water soluble form, of toxic nature to virulent human tubercle bacilli, capable of abolishing their infective power for guinea pigs within a short time (within about 20 hours at 37° C.). The final hydrogen-ion concentration of the mixture plays no part in its toxicity to tubercle bacilli. Different normal tissues form the tuberculo-toxic substance in different strength, voluntary muscle being apparently the more active, the toxicity of its filtrate being sufficient to abolish the virulence of tubercle bacilli in one day; liver, lung and spleen accomplishing the same within 4 days; brain, heart muscle and kidney only after one week (brain frequently produced an inactive filtrate). The tuberculo-toxic substance is thermostabile and is not removed by prolonged aeration. The tuberculo-toxic substance is probably identical with the tissue autolysates which Conradi in 1902 found bactericidal to the rapidly growing microorganisms.

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